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BINDING ASSAYS FOR THE QUANTITATIVE DETECTION OF P. BREVIS POLYETHER NEUROTOXINS IN BIOLOGICAL SAMPLES AND ANTIBODIES AS THERAPEUTIC AIDS FOR POLYETHER MARINE INTOXICATION

FINAL REPORT

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The polyether lipid-soluble toxins isolated from the marine dinoflagellate *Ptychodiscus brevis* can be detected using immunoassays. Brevetoxins can be covalently linked to proteins to produce fully antigenic materials. Antigen can be used to raise antibodies in goats using the toxin-protein conjugates. Both radioimmunoassays and enzyme-linked immunoassays can be developed using the goat antibodies. Toxin derivatives in which enzymes are covalently attached are unstable and lose enzymatic activity under all conditions examined. Toxin antibody purification can be aided using covalent toxin affinity columns employing aminohexyl Sepharose as solid support. Immunoassays show no cross-reactivity with okadaic acid or the organisms responsible for its biosynthesis but all brevetoxins can be detected, including crude toxin in extracts of *P.* cells or in the adsorbed cells themselves in enzyme-linked formats. Ciguatoxin (or fish flesh toxins) can be detected as well, although the quantitative nature of the interaction is still unknown. Antibrevetoxin antibodies can be

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used in vitro to either compete for toxin with synaptosomes, or to remove toxin from synaptosomes once they have adsorbed. Successful immunoprophylaxis can be anticipated using approriate regimes. Fully developed enzyme-linked immunoassays for the detection and quantification of brevetoxin-like polyether toxins in biological samples is anticipated within a year.

FOREWORD

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

In conducting the research described in this report, the investigator has adhered to the "Guide for the Care and Use of Laboratory Animals", prepared by the committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication (NIH) 86-23, revised 1985)).

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I. Statement of the Problem

This contract is concerned with the development of diagnostic methods and therapy for exposure to polyether toxins produced by marine dinoflagellates. We have proposed two separate binding assays which have potential value in quantitative detection of these toxins in biological samples. Our specific aims are to:

[1] develop and refine *in vitro* radiometric binding assays to detect polyether marine neurotoxins in biological samples using tritiated brevetoxin PbTx-3 (formerly T17) as radiometric probe and employing antibodies prepared in goat against PbTx-3 produced by laboratory cultures of *Ptychodiscus brevis* or synaptosomes prepared from rat brain;

[2] determine the sensitivity and specificity of the binding assays using brevetoxin standards mixed with biological samples of clinically-obtainable types, i.e. serum, mucousal secretions, urine and or feces;

[3] using goat antibodies or solubilized brevetoxin binding component from rat brain, develop enzyme-linked assays to further simplify the procedure for routine use;

[4] examine potential cross-reactivity of the binding assays with respect to other polyether toxins, and hence their usefullness in the detection of other lipid-soluble marine polyether toxins;

[5] examine the feasibility of using available antibodies as therapeutic agents, first using competitive *in vitro* molecular pharmacological binding assays, and later by examining the reversal of toxic effects in animals by immunoassay;

[6] provide reagents adequate for 10,000 assays, including radioactive toxin probe, and data on tests and evaluations. Detailed protocols will accompany reagents.

II. Summary of Results Generated

A. Deliverables

1. Goat Polyclonal Antibody [Deliverable Item 0001AA]: Polyclonal antibody is available prepared against two different brevetoxin-protein conjugates, bovine serum albumin and keyhole limpet hemocyanin. These materials are stored as described in 2. below, are available upon request, and exceed the amount contracted. Prepared material represents approximately 4 liters of serum and the animals continue to be boosted and serum collected.

2. Detailed Assay Protocol for Item 0001AA [Deliverable Item 0001AB]: We continue to boost a single goat with KLH-toxin conjugate on alternate weekly intervals, with bleeds on the opposite weeks to immunization. The week of 15 May 1990 is the final week in the innoculation series, and the animal will be allowed to rest for several months following this boost. Titers will be monitored weekly and the animal will be plasmaphoresed at peak titer.

The BSA-animal has been maintained but not boosted for six months. Immunization protocols will begin 15 May 1990, a titers will be monitored one week following the boost. Serum will be obtained upon attainment of optimum titers.

For IgG purification, bleeds are allowed to clot, and the serum is separated by centrifugation. Antisera are traditionally treated with 0.5 volumes of saturated ammonium sulfate under conditions of stirring, and are allowed to precipitate overnight at 4°C. Precipitates are removed by centrifugation at 3000 x g and the supernatant solutions are brought to 50% ammonium sulfate saturation. Precipitate (IgG) is retained , redissolved in 0.3 volumes of the original volume

in phosphate buffered saline containing 0.01% sodium azide. IgG is stored for longer periods of time, lyophilized from distilled water, and is reconstituted in PBS prior to use.

Alternatively, a method now being explored is isolation of antibrevetoxin antibodies using a batch column recombinant DNA Protein G column, manufactured by Genex Corporation. This genetically engineered product is devoid of the serum albumin binding portion, and thus can be used for direct IgG purification from serum. The column we are using contains 50 ml Gammabind™ Plus gel, capacity for goat IgG of about 1.11 grams per run. We estimate we shall be able to save approximately 75% of our effort in IgG purification using this procedure.

3. Assay Reagents for Synaptosome [Deliverable Item 0001AC]: Synaptosomes are prepared in multiple runs, frequently 10-50 brains per run. Frozen brains were purchased from Harlan Sprague Dawley company in multiples of 50-200 brains, and were stored at -80°C until use. Synaptosomes were prepared according to the method of Dodd *et al.* (1) and were stored as pooled samples from 200 brains. This allows for a reproducible preparation lot for several weeks work. These materials continue to be prepared at approximately 2 week intervals as needed, and are available when requested. Reagent availability exceeds that amount contracted.

4. Detailed Assay Protocol for Item 0001AC [Deliverable Item 0001AD]: Binding of tritiated toxin is measured using a rapid centrifugation technique (2). Binding assays are performed in a binding medium consisting of 50 mM HEPES (pH 7.4), 130 mM choline chloride, 5.5 mM glucose, 0.8 mM magnesium chloride, 5.4 mM potassium chloride, 1 mg/mL BSA, and 0.01% Emulphor EL-620 as an emulsifier for toxin.

Synaptosomes (40-80 μg total protein), suspended in 0.1 mL binding medium minus BSA were added to a reaction mixture containing tritiated PbTx-3 and other effectors in 0.9 mL binding medium in 1.5 mL polypropylene microcentrifuge tubes. After mixing and incubating at 4°C for 1 hour, samples were centrifuged (15K x g) for 2 minutes. Supernatant solution was aspirated from each tube and the pellets were rapidly washed with several drops of a wash medium (3). Pellets were then transferred to liquid scintillation vials and bound radioactivity was measured. Non-specific binding was measured in the presence of 10 μM PbTx-3 and was substracted from total binding to yield a calculated measure of specific binding. Free tritiated probe was measured by counting an aliquot of supernatant solution prior to aspiration.

5. Radioactive Brevetoxin Probe Tritiated PbTx-3 > 8 Ci/mmole [Deliverable Item 0001AE]: Tritiated PbTx-3 is continually available at specific activities ranging from 10-25 Ci/mmole, wholley dependent upon the specific activity of the sodium borotritiide used for reduction. Using 1 Ci of tritiated sodium borotritiide, we have been successful in producing between 200 and 250 mCi of purified brevetoxin PbTx-3, and minor amounts of tritiated brevetoxin PbTx-9. This item is complete and exceeds the amount requested.

6. Reagent for Enzyme-Linked Assays [Deliverable Item 0001AF] The reagents required for the enzyme linked immunoassay include goat-antibrevetoxin-IgG, Blotto blocking reagent, rabbit-antigoat IgG linked to peroxidase, and ABTS substrate. The first reagent is available from Items 0001AA and 0001AJ, the second is basically buffered non-fat dry milk, and the latter two reagents are purchased reagents available from a number of commercial sources. We nominally chose Pierce Chemical Company, largely on the basis of lot-to-lot reproducibility, and availability. These materials are all available is excess of the contracted amount.

7. T34 (PbTx-2) and T17 (PbTx-3) Brevetoxins in Culture Ratio of 3:1, 10 mg/month of Homogeneous Toxins [Deliverable 0001AG]: This Deliverable is complete, and exceeded the 360 mg total toxin amount contracted.

8. Approximately 500 μg Tritiated T17 (PbTx-3) Brevetoxin at Specific Activity of 8-20 Ci/mmole, Labeled at C-42 [Deliverable Item 0001AH]: Two hundred micrograms of the contracted 500 μg have been supplied. We anticipate requests for the remaining material during the remainder of calendar year 1990, and agree to supply the remainder as needed by USAMRIID. The material has a nominal shelf life of three months before repurification is required, and we can accomplish re-purification readily. Material up to an additional 300 μg tritiated PbTx-3 will be supplied as needed.

9. Goat Immune Serum Prepared Against BSA-hapten Conjugate T17 (PbTx-3) Toxin [Deliverable 0001AJ]: Additional amounts of goat immune serum were requested and we can comply with additional material. Serum available within USAMRIID is of higher titer than is our material. This Deliverable item is a duplication of Item 0001AA, as crude serum, and is available when requested.

10. 500 micrograms of T17 (PbTx-3) Protein Conjugate [Deliverable 0001AK]: This item is complete, supplied as a BSA conjugate with an approximate 10.3:1 (Toxin:Protein) ratio. There are no further Deliverables specified in the contract.

B. Additional Reagents

1. Toxin-Protein Conjugate for Immunization. Both radioimmunoassays and enzyme-linked immunoassays utilize specific antibody against brevetoxin PbTx-3. Complete antigen construction has been previously investigated using either bovine serum albumin-covalently linked to brevetoxin, or more recently we have used keyhole limpet hemocyanin-linked brevetoxin. For this year's annual report (4), all of our work has utilized brevetoxin covalently linked to KLH as complete antigen. This overcomes many of our initial problems with cross-reactivity due to small amounts of BSA present in many of the assays, and KLH also elicits better antibody responses in animals.

Purified PbTx-3 was dissolved in minimal redistilled pyridine, and was succinylated with 10-fold molar excess succinic anhydride as previously described. Following separation of unreacted PbTx-3 and succinic anhydride from toxin-succinate using TLC (70/30 ethyl acetate/light petroleum), the free carboxyl function on the conjugate was covalently coupled to the ϵ-amino function of lysyl residues on the KLH using standard procedures (5). Following coupling, the mixture was dialyzed against PBS, pH 7.4 for overnight, and the protein concentration adjusted to yield "toxin equivalents" of 1 mg/mL.

2. Toxins. Natural toxins were utilized as obtained. Brevetoxins were purified from laboratory cultures of *Ptychodiscus brevis*, okadaic acid was obtained from Dr. Robert Dickey at the FDA Dauphin Island laboratory, and ciguatoxic fish flesh was supplied by Dr. Thomas Tosteson at the University of Puerto Rico. Synthetic tritiated PbTx-3 was produced from PbTx-2 by chemical reduction employing cerium chloride and sodium borotritiide. Crude PbTx-3 was purified using reverse phase high performance liquid chromatography. HPLC-purified toxin had demonstrated specific acitivities of 10-15 Ci/mmole.

3. Derivatized Toxins. Brevetoxin PbTx-3 was linked to horse radish peroxidase, and the procedure was optimized, both with respect to reaction conditions and to stoichiometry. Derivatized materials were evaluated for stability. Attempts to link PbTx-3 to chloroperoxidase were also evaluated. PbTx-3 was also linked to urease and toxin-enzyme conjugates were evaluated.

4. Toxin Enzyme Conjugates. Brevetoxins linked to either Jack Bean urease or Horse Radish peroxidase were evaluated as specific probes in

immunoassays. These toxin-enzyme conjugates were prepared as succinate linked materials as described in materials and methods. For urease conjugates, 1:2, 1:4 and 1:6 molar ratios were investigated. The following protocol yielded the most reproducible results:

[1] prepare succinylated PbTx-3 as previously described;

[2] dissolve succinylated PbTx-3 in minimal 50% pyridine and add 2 mg EDC carbodiimide for every mg succinylated toxin (about 10-fold excess EDC);

[3] after 2 hr mixing at room temperature, add succinate toxin to a 5.7-fold excess urease in 1 volume equivalent of water, in three equal aliquots. Total volume after addition is 2-fold the volume in step [2], final pyridine concentration 25%;

[4] stir at room temperature overnight;

[5] transfer to dialysis tubing (MW exclusion 10,000) and dialyze against 3 changes of pH 7.4 phosphate buffered saline.

For peroxidase conjugates, toxin:enzyme molar ratios of 1:1, 1:2, 1:4, and 1:6 were evaluated. The following protocol yielded the most reproducible results:

[1] prepare succinylated toxin as previously described;

[2] dissolve succinylated PbTx-3 in minimal 50% pyridine and add a 2:1 mg ratio of EDC carbodiimide relative to toxin (again a 10-fold excess);

[3] stir at room temperature overnight;

[4] after mixing overnight, mix enough horse radish peroxidase in water to so that when the toxin succinate is added, the final ratio of 1:6 molar toxin:HRP coupling mixture (0.25 mg toxin succinate to 90 mg HRP) will be achieved;

[5] stir at room temperature 2 hours and at 4°C overnight;

[6] dialyze as in urease assay.

In all cases it is imperative to add toxin-succinate- carbodiimide <u>to</u> enzyme and not enzyme to hapten. In this manner, maximum enzyme activity is maintained by limiting the number of haptens linked per enzyme.

5. Brevetoxin Affinity Column. Brevetoxin affinity column are constructed using aminohexyl Sepharose as solid support, and linking brevetoxin PbTx-3-succinate derivative to it using standard carbodiimide coupling procedures. This procedure, carried out in 50% pyridine solution, results in toxin succinate covalent binding via. peptide linkages to the amino function of the AH Sepharose at about a 75% coupling efficiency and a specific binding capacity of 1-6 μmoles of toxin bound per mL of swollen gel. We utilize lower coupling stoiciometries to aid in ease of handling 1-5 mg of toxin solid support conjugate (2-10 mL of gel) rather than high stoichiometries which yields smaller amounts of gel (on the order of 0.2-1.0 mL gel). This column matrix is useful for antibody purification following protein G purification.

Virtually all of the reagents and derivatives described or summarized herein are unique materials which were conceived and synthesized under support from USAMRIID. Use of these individual reagents by both our laboratory and USAMRIID indicate that most work as anticipated. Below, we summarize the usefullness and stability of each of the reagents, or assays developed using individual reagents in different formats. Two of the formats are useful in detecting and quantifying toxin in biological matrices, and are also applicable to routine clinical detection for assessment of potential exposure to these marine toxins.

C. Stability of Reagents

1. Synaptosomes. For experimental work to proceed utilizing an homogeneous and reproducible preparation, we developed a procedure which allows for the preparation of 20-50 rat brains, storage of synaptosomes at -80°C, and use over a period of days to weeks of small aliquots of synaptosome preparation. The availability of whole unstripped rat brain from Harlan Sprague Dawley Inc in Indiana allowed us to work-up approximately 6-8 times the number of rat brains at the same over-all cost. Brains are worked up according to the method of Dodd *et al.* (1), and are stored as synaptosomes in serum binding medium SBM (5) in 5 mL aliquots. Procedurally, for each brain prepared (10 mL total final volume), 0.5 mL of the preparation is aliquoted into 20 tubes and frozen. Each group of brains is prepared in this manner, adding 0.5 mL equivalents per brain to each of the twenty tubes. When tube volumes reach the 5 mL volume, the aliquots are stored as a batch of twenty tubes, each of 5 mL volume and equivalent in sample. Each tube, when thawed for use, will allow for the preparation of a "two-tray" assay of either standard displacement or competition type. Using this procedure, we are able to reproduce results within a batch with very low standard errors and deviation, and batch to batch variation is likewise very low. We could detect no change in either dissociation constant or binding maximum using synaptosomes prepared in this manner, when compared with freshly prepared material. We have been unsuccessful in utilizing detergent-solubilized brevetoxin binding site as an assay component.

2. Antibodies. Following ammonium sulfate precipitation, dialysis against distilled water, and lyophilization, anti-brevetoxin IgG fraction is stable indefinitely at -20°C in sealed serum bottles. We have been able to detect no difference in specific tritiated brevetoxin binding with age of stored material. Lyophilized material theoretically should be stable at room temperature so prepared, but because of the limited amounts of specific antibody available the study does not presently warrant investigation. Preparation of antibody to this step allows for an approximately 15-fold purification, and a reduction in storage volume from 4 liters to 500 mL in ten separate bottles.

From crude serum, which exhibited about 15 units specific brevetoxin binding equivalents per milligram protein, and ammonium sulfate precipitation which increased specific binding to 158.8 units per milligram protein, we subjected antibrevetoxin antibody to sequential protein G- and brevetoxin-specific affinity chromatography. Overall, 29.5% of the specific binding equivlanets were recovered through purification, with a final specific binding of 307 units per milligram protein.

Regardless of the short-comings in specific IgG purification, and loss of some toxin binding activity, material which is specifically adsorbed and desorbed from brevetoxin affinity columns is substantially better for development of microtiter plate assays, as will be demonstrated later in this report. The specific antibody is also an excellent reagent for locating brevetoxin photoaffinity probes on voltage-sensitive sodium channels, following SDS gel electrophoresis and Western blotting (6). Specific binding affinity ranges from 80-90 % in purified preparations. The dissociation constant for brevetoxin-specific antibody is 1.32 nM, with an exhibited binding maximum of 17.7 pmoles/mg protein in crude serum (2.8 μmole toxin/mmole antibody) or about 58 μmoles toxin/mmole antibody in brevetoxin affinity column purified material.

The low titers we achieved were partially remedied by purification of the specific antibrevetoxin antibodies and concentration of that fraction of serum. The avidity of the complex, i.e. the overall stability of the antibody-antigen interaction, is rather low, which is exploitable in terms of competition assays for toxin detection. Radioactive toxin-antibody complexes can be easily

perturbed by unlabeled toxin, whether the unlabeled material is added at the same time as the radioisotope, or later after complex formation. This allows for the development of true "displacement" assays, as opposed to the more conventional "competition assays".

3. Enzyme-Conjugates. Toxin-urease conjugates lose activity with time, even in the refrigerator or at freezer temperatures. Several sequential batches of toxin-urease (1:5.7) became inactive (no enzyme activity but still protein and toxin present at proper stoichiometry) very quickly and within a couple of weeks exhibited less than 10% of the assayed activity at the time of synthesis and dialysis. We are uncertain as to the cause of the inactivation. Based on our previous experience with uncoupled urease and its sensitivity to heavy metals and temperature regimes, we initially began to develop and utilize reagent buffers which contained low concentrations of heavy metals (i.e. reagent or ACS grade or better). However, we began to doubt the utility of an urease assay, especially when considering the various and multiple contaminants which might be present in actual samples. Aside from figure 7 reported in Year 2 Annual Summary Report (7), we report no further on urease-toxin assays. Thus, with this enzyme system we were unsuccessful in our attempt, even though we could demonstrate a dose-dependent displacement of toxin-enzyme conjugate while conjugates remained active (8).

Assays employing HRP were much more readily adaptable and useable owing to the inherent stability of peroxidase conjugates. Our initial results using toxin-peroxidase in 50% glycerol were not encouraging, and much of the enzyme activity was lost in a period of days, as in the urease case without loss of either protein or toxin. Thus, something was happening to the enzymatic activity. Six different storage protocols for toxin-enzyme conjugate were tested: [1] speed vacuum-dried (Savant) preparation with 0.1% BSA, frozen and dessicated; [2] speed vacuum-dried with no BSA, frozen and dessicated; [3] speed vacuum-dried with 0.5% BSA, room temperature dessicated; [4] speed vacuum dried with no BSA at room temperature dessicated; [5] frozen in solution with 0.1% BSA; and [6] frozen with 0.1% BSA plus 50% glycerol.

Based on recovery of enzymatic activity, protocol [3] was best for maintaining integrity of the preparation, followed by [5] and [6]. These recoveries indicate that during coupling of toxin to peroxidase, approximately 40% of the peroxidase activity is retained.

Following storage under conditions of [3], 15% SDS polyacrylamide gel electrophoresis was performed on the derivatized toxin-enzyme, and the migration was compared with that of unconjugated toxin or enzyme. Coomassie brilliant blue staining of developed gels indicated unreacted HRP to possess a molecular radius of about 44,000 Daltons. Unconjugated toxin did not visualize with staining--as was expected. Toxin-peroxidase conjugates possess a molecular radius of about 48,000 to 54,000 Daltons, with no 44,000 molecular weight material remaining. Thus, we are certain we have conjugated the toxin to the enzyme, and believe that the 40% activity retained is due to a 60% reduction in enzymatic activity caused by toxin conjugation---and not due to 100% reduction in 60% of the enzyme due to conjugation, with the remaining 40% activity arising from that portion of the enzyme preparation not conjugated. This point is <u>critical</u> to assay development, and has been demonstrated.

Western blotting of the SDS gel, and subsequent ABTS substrate incubation, indicates that the peroxidase with altered mobility on gels is indeed still active. This fact, when compared with gels cut up and assayed for tritium counts associated with added tracer PbTx-3 toxin, and silver stained Western blots, indicates that enzyme activity, toxin, and protein all comigrate in SDS gels and are distinct from unlatered enzyme or toxin.

Antibrevetoxin antibody-peroxidase conjugates are a conventional reagent, in deference to those already outlined. The antitoxin-peroxidase conjugate was determined to be very stable,on the order of the stability of the enzyme itself. Strong signals in ELISA's (next section) indicated about 1:1 conjugation ratio for antibody to enzyme and allowed for a 1:20,000 diltuion of this reagent. Yields for this periodate conjugation reaction were low however, adjudged to be about 35%, and usefullness of the reagent was directly correlated with the purity of the antibody utilized for coupling. Only ammonium sulfate precipitated antibody had been used for coupling, and at that time high quality HRP-linked rabbit antigoat antibody became available. Thus although the feasibility is evident for antibrevetoxin-peroxidase probes, the use of antispecies-peroxidase conjugates allows for reproducibility and convenience, especially when coupled with the amplification possible with sandwich assays. Antispecies antibodies linked to enzymes have been extremely stable in our hands.

D. Cross-Reactivity of Polyether Toxins with Brevetoxin Assays.

1. Immunoassays. In our previous Annual Reports, we described the interaction of antibrevetoxin antibodies with the suite of naturally-occurring and synthetic brevetoxins at our disposal. We described the higher sensitivity of detection of the PbTx-2 type backbone versus PbTx-1 type backbone, and postulated a bit about the potential for cross-reactivity with other polyether dinoflagellate toxins like ciguatoxin and okadaic acid. However, those materials were unavailable to us, and we had no prospect of obtaining them. In fact, some excellent work has been done by Dr. Mark Poli at USAMRIID using antibrevetoxin antibodies and several different types of marine polyether toxins which he obtained from collaborators (9).

Neither okadaic acid, supplied by Dr. Robert Dickey (FDA Dauphin Island Laboratory) in purified form, nor the progenitor organism *Prorocentrum lima*, supplied by Dr. Carmelo Tomas (Florida Department of Natural Resources Marine Research Institute), reduced the specific binding of brevetoxin to its specific antibody binding site. Nor did sandwich ELISA brevetoxin immunoassays cross-react with okadaic acid or the organisms in micotiter plate assays.

Crude ciguatoxin, isolated from ciguatoxic barracuda and supplied by Dr. Thomas Tosteson of the University of Puerto Rico, was subjected to radioimmunoassay following high performance liquid chromatography; 10 minute fractions were collected for 60 minutes and were correlated with intraperitoneal and oral mouse bioassay. and synaptosome assay (Figure 1).

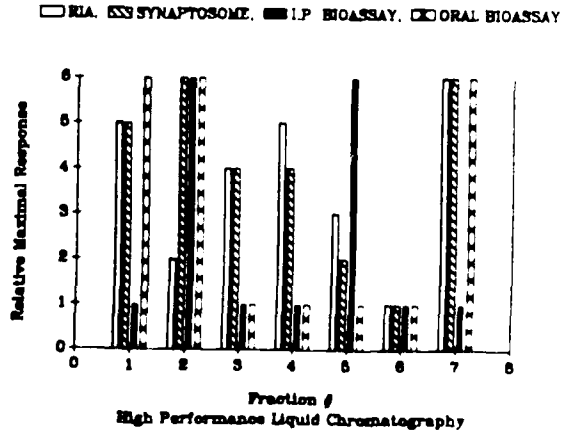

Figure 1. Correlation of immunoassays with mouse bioassay and synaptosome assay.

This material did cross-react, and two of the orally potent fractions (defined as "ciguatoxin") produced strong correlations.

E. Disposition of Enzyme-Linked Immunoassays

Seven different ELISA protocols were explored during the contract period. These are summarized in Table I. Of the assay protocols explored, only #7 was deemed worthy of further work.

Table I. Summary of Brevetoxin Microtiter Plate Assays

Adsorbant			Sensitivity (ng/well)
Primary	Secondary	Tertiary	
1. Toxin	IgG α PbTx-3	Protein A-urease	1.0
2. IgG α PbTx-3	PbTx-3-urease	----------	0.001
3. KLH-PbTx-3	IgG α PbTx-3	rabbit α goat IgG-Peroxidase	0.2
4. Synaptosome	PbTx-3-urease	----------	0.2
5. IgG α PbTx-3	PbTx-3-peroxidase	----------	0.001
6. Synaptosome	PbTx-3	IgG-peroxidase α PbTx-3	-----
7. Toxin	IgG α PbTx-3	rabbit α goat IgG-Peroxidase	0.040

Protocols # 1 and #7 are capable of detecting toxin by a non-competitive direct format. Increasing color development of enzyme cleaved substrate is in direct correlation with toxin content in unknowns. Protocols #2, #3, #4, #5, and #6 are competitive protocols in which unknown toxin is added following secondary adsorbant and prior to tertiary adsorbant. Color development in these latter cases is inversely proportional to toxin concentration in unknowns.

Protocol #1 was discarded because of low sensitivity due to instability of urease enzyme and conjugates, and because of low binding affinity of Protein A for goat IgG. Protocol #2 was discarded because of high background, low affinity of Protein A for goat IgG, and low activity of toxin-enzyme conjugate. Protocol #3 was discarded because of its competitive assay type, but not before we noted that the "sandwich nature" of the assay, i.e. detecting antibrevetoxin IgG using an antispecies antibody enzyme conjugate, increased sensitivity by allowing formation of multimer complexes of antibody-enzyme. Assay #4 was discarded because of the urease problems already outlined. Protocol #5 was not used because of low activity of toxin-peroxidase conjugate and competitive nature. Protocol #6 was discarded because of troubles with solubilized synaptosomes and specific binding.

Only assay #7 was exlored further and refined. Toxin is assayed directly in standards or unknowns by first non-specifically adsorbing hydrophobic toxin to wells of plates, followed by blotto blocking and washing. Addition of antibrevetoxin antibody and incubation, followed by washing yields goat antibrevetoxin specifically adsorbed to toxin stuck on the plate. From this step onwards, all reagents are commercially available---a fact which is a decided plus in these assays. One only needs specific antibrevetoxin antibody and standards for the assay to be of value. Rabbit antigoat-peroxidase conjugate is available in high titer and high peroxidase specific activity. ABTS substrate is also commercially available and makes assay development relatively easy. Rabbit antigoat conjugates are also available with alkaline phosphatase enzyme

conjugated, should assays with peroxidase pose problems with high inherent peroxidases in the sample being assayed.

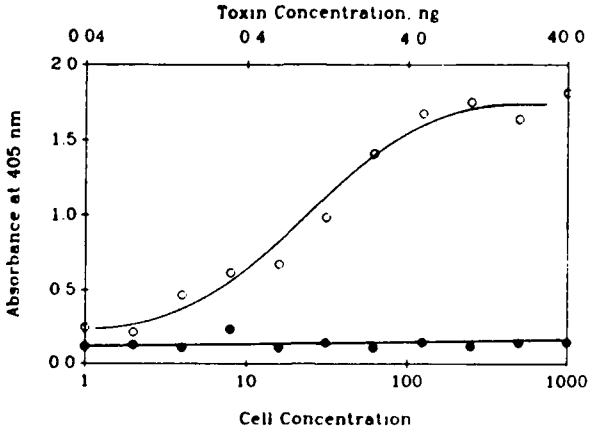

Figure 2. Non-Competitive Peroxidase-Linked Sandwich Immunoassay. Assays were conducted at room temperature, measuring ABTS substrate conversion at 405 nm according to the protocol listed illustrated in #7 of Table I. Samples of *F. brevis* cells, *Prorocentrum lima* cells, okadaic acid, or brevetoxin PbTx-3 were incubated 1 hour as primary adsorbants. Following a 1 hr Blotto blocking of non-specific binding sites, 30 μg antibrevetoxin protein G purified IgG was added to each well for a 1 hr incubation. Rabbit anti-goat IgG linked to peroxidase was added as tertiary adsorbant (1:1000 to 1:5000 diltuion of commercial preparation). After addition of ABTS substrate, changes in absorbance were measured for a two hour period, over which period color development was linear. A derived value per hour was calculated. Cell concentrations for dinoflagellates are quantified on the lower X-axis, and toxin concentrations are quantified on the upper X-axis. Brevetoxins and *P. brevis* cell extracts are illutrated by the open circles. Okadaic acid and *P. lima* cell extracts are illustrated in closed circles.

Sandwich assays are being refined under separate funding from the Florida High Technology Council. The results of work performed under the Florida High Technology grant during calendar year 1990 will be forwarded to USAMRDC in 1991, as an Appendix to this Final Report.

F. Immunotherapeutic Intervention *In Vitro*

As stated earlier in this Final Report, the dissociation constant (K_d) for toxin binding to antibody is 1.32 nM, i.e. a higher affinity than the 2.6 nM dissociation constant measured for synaptosomes. Theoretically, antibody should compete for free toxin in solution, when both synaptosomes and antibody are present. Studies carried out in which increasing amounts of antibody are added to synaptosome binding experiments in the presence of constant radioactive toxin indicate that at IgG:synaptosome binding ratios of 1:1, approximately 50% of the toxin normally bound to synaptosomes is competitively bound to IgG instead. Neither pre-immune serum nor serum albumin exhibits this effect on synaptosomes (Figure 3 a and b). If synaptosomes are pre-incubated one hour with

tritiated toxin prior to adding IgG, an identical effect is observed. That is to say, IgG is capable of displacing the tritiated toxin from its specific site of binding on synaptosomes (Figure 3 c). Thus, it appears that immunotherapeutic intervention may be possible *in vivo*, a finding in agreement with work done at USAMRIID by Poli et al (10).

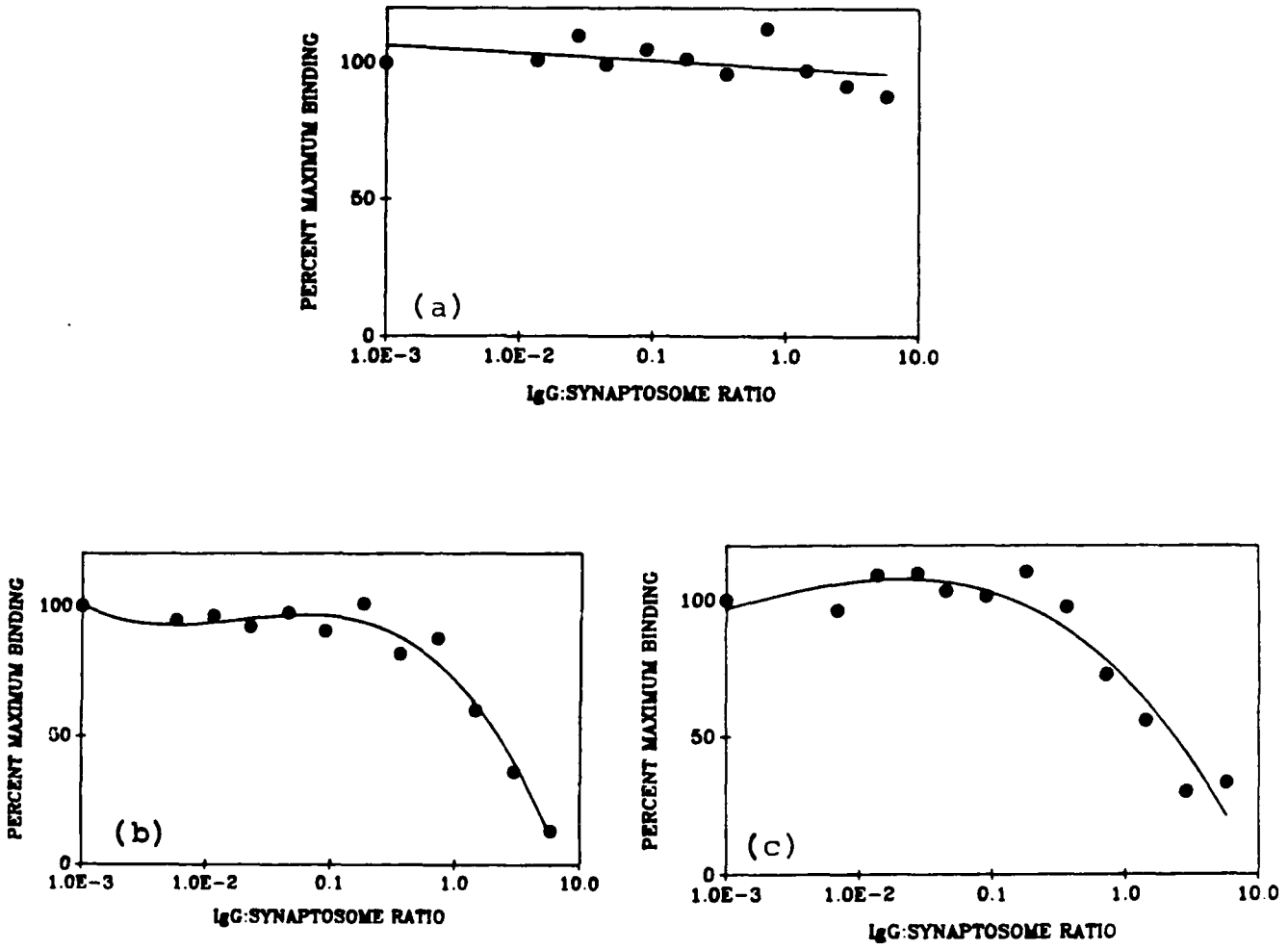

Figure 3. Binding of tritiated brevetoxin to synaptosomes and antibody: competitive versus displacement affinities. 2.0 nM tritiated PbTx-3 at 13.4 Ci/mmole; synaptosomes at 300 μg protein; IgG 0.18-1800 μg protein. Incubations were carried out at 4°C for one hour, followed by centrifugation and estimation of tritiated PbTx-3 in synaptosomal pellets by liquid scintillation counting. (a) IgG represents pre-immune serum; (b) and (c) IgG represents immune serum, dissociation constant 1.3 nM. IgG:synaptosome ratio calculated from known binding maxima in each case. (b) is a competition study with IgG and synaptosomes added together with tritiated toxin. (c) represents pre-incubation with synaptosomes and toxin, followed after 1 hour by IgG addition.

III. Conclusions

[1] Brevetoxin can be covalently linked to proteins BSA or KLH to produce fully antigenic materials;

[2] Antibodies can be raised in goats using the antigenic brevetoxin-protein conjugates;

[3] Radioimmunoassays using the elicited antibodies and tritiated brevetoxin PbTx-3 can be developed;

[4] Immunoassays can be converted from a radiometric format to an enzyme-linked format;

[5] Derivatization of toxin to an enzyme-linked conjugate confers a lowered stability to the enzyme coupled;

[6] Toxin can be immobilized on aminohexyl Sepharose to create toxin affinity column for antibody purification;

[7] Enzyme-linked immunoassays protocols using toxin (in unknowns or standards) as primary adsorbant on microtiter plates, goat antibrevetoxin as secondary adsorbant, and rabbit antigoat IgG linked to peroxidase, detect brevetoxin in quantities as low as 0.04 ng/well;

[8] There is no cross-reactivity from okadaic acid in either RIA or ELISA;

[9] Toxins from fish flesh (ciguatoxin?) can be detected using the immunoassays;

[10] Fully developed ELISA protocols and kits are anticipated by 12/90;

[11] Antibrevetoxin can effectively compete with synaptosomes for tritiated brevetoxin *in vitro*, and once bound to synaptosomes the antibodies are effective in removing toxin from the synaptosomes.

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VI. Personnel Receiving Contract Support

A. Professional Personnel

Daniel G. Baden, Ph.D. P.I. [entire tenure of contract] 35% effort
Thomas J. Mende, Ph.D. Investigator [entire tenure of contract] 10% effort

B. Students

Vera L. Trainer Ph.D. Student [entire tenure of contract] 100% effort
Richard A. Edwards Ph.D Student [entire tenure of contract] 100% effort
Michelle Zetwo, Undergraduate Work-Study [6/11/88-8/19/88, 5/13/89-6/30/89] approximately 10 hours per week.

C. Staff

Laurie E. Roszell [1/9/87-8/17/89] 100%
Lloyd Schulman [8/18/89-11/30/89] 100%

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